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(54) Title: A METHOD AND A KIT FOR THE DIAGNOSIS OF IgA NEPHROPATHY

(57) Abstract

A method for the diagnosis of IgA nephropathy using an antigene-antibody interaction comprising the steps: a) preparing a substrate capable of binding fibronectin or IgA, b) contacting the substrate resulting from step a) with a sample of body fluid drawn from a patient subject to diagnosis to bind any fibronectin-IgA-complex present in said sample to the substrate, and c) determining the presence of complex bound to the substrate using the reaction between the exposed part of such bound complex and a corresponding antibody thereto; and a diagnostic kit for use in such diagnosis.

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# A method and a kit for the diagnosis of IgA nephropathy.

The present invention relates to a method for the diagnosis of IgA nephropathy using an antigen -antibody interaction, and the invention also includes a diagnostic kit for use in such a diagnosis.

Patients with primary IgA nephropathy, also known as Berger's disease, have been shown to have circulating IgA antibodies, binding to collagen IV prepared from glomerular basement membrane (GBM) (1). It was also shown that the IgA antibodies bound equally well to collagen I,II and IV and that denatured collagens bound antibodies most efficiently (1).

In view of the wide distribution of the various collagens, it is of interest to note the coexistence with IgA nephropathy of symptoms from extrarenal organs (2). Thus symptoms from skin, eye and joints are of particular interest since these structures contains collagen as a major component. Indeed one study reports presence of vascular IgA deposits in skin from patients with IgA nephropathy (3).

The association of exacerbations of clinical disease with upper respiratory tract or gastrointestinal infections and the finding of IgA deposits in the glomerular mesangium is well known. The finding of increased levels of IgA-bearing peripheral lymphocytes (4) as well as decreased IgA-specific suppressor T cell activity (5) and increased IgA-specific helper T-alfa cells (6) suggests an immunological mechanism.

Fibronectin, also known as cold insoluble globulin (CIg), that is altered in many disease processes, is present both as a plasmaprotein and as a cell surface protein. The two forms differ slightly in composition but share important functions as binding to gelatin (collagen), heparin, fibrin and cell surface receptors (7). Fibronectin is the major plasma component binding to gelatin (8) and this property as well as its heparin binding properties have been utilized in its isolation (9). Interestingly fibronectin is present also in basement membranes. It can be

visualised by immunofluorescense not only in the glomerular basement membrane but also in the mesangium of healthy individuals (10). Furthermore a concomitant increase of mesangial fibronectin and mesangial matrix was observed in patients with IgA nephropathy and Henoch-Schönleins purpura (10).

The present invention is based on the surprising discovery, that the IgA-antibodies are present in circulating immune complexes in patients with primary IgA nephropathy together with fibronectin, and this unexpected finding does, of course, explain the fact that the igA-containing complexes have the ability of binding to collagen. This new finding that the circulating immune complex contains, in addition to IgA, also fibronectin, enables the provision of both method for the diagnosis of IgA nephropathy and a diagnostic kit for use in such diagnosis.

Accordingly, one object of the present invention is to provide a method for the diagnosis of IgA nephropathy using antigen-antibody interaction, and another object of the invention is to provide a diagnostic kit for use in such diagnosis of IgA nephropathy.

The method of the present invention is characterized by the following steps:

a) preparing a substrate capable of binding fibronectin or IgA,

b) contacting the substrate resulting from step a) with a sample of body fluid drawn from a patient subject to diagnosis to bind any fibronectin-IgA-complex present in said sample to the substrate, and

c) determining the presence of complex bound to the substrate using the reaction between the exposed part of such bound complex and a corresponding antibody thereto.

In preparing such substrate capable of binding fibronectin preferred binding agents are collagen, heparin, fibrin or anti-fibronectin. According to this aspect of the invention the substrate is prepared in such a way as to bind the fibronectin-IgA-complex through its fibronectin component. This in turn means that the IgA-component of the complex is

available for determining the presence of complex bound to the substrate.

On the other hand the binding of the fibronectin-IgA--complex to the substrate can be directed to the IgA-part of the complex. In such case there can be used as a binding agent either anti-IgA or a lectin, both of which are capable of binding IgA. In this case the fibronectin part of the complex will be available for determining the presence of complex bound to the substrate.

The determination under step c) of the method of this invention can be of a quantitative nature and can be based on a conventional detection system. Among such detection systems there may be mentioned those based on enzymatic activity, or systems based on radiation emitted by a radioactive isotope or based on fluorescence.

The substrate used in binding the fibronectin-IgA-complex present in a sample subject to diagnosis can be constituted by different objects, such as microtiter plates, laboratory test tubes, nitro-cellulose paper, plastic spheres or any other object suitable for the purpose.

Samples of body fluid drawn from a patient subject to the diagnostic method of the present invention may be constituted by for example human blood, serum, plasma or saliva.

The invention also provides for a diagnostic kit for use in the diagnosis of IgA nephropathy, and such kit comprises:

a) an object capable of binding fibronectin or IgA, and

b) reagent comprising a component which is capable of binding IgA or fibronectin and a detection component enabling determination of fibronectin-IgA complex bound to the said object.

In the embodiments wherein the IgA part constitutes exposed part of the complex the object carries attached to the surface thereof heparin, collagen, fibrin or anti-fibronectin. In such case the reagent preferably comprises lectin or antibody directed against IgA.

In the opposite case, wherein fibronectin is the exposed part of the complex the object carries attached to the surface thereof anti-IgA or lectin, the reagent comprising heparin, collagen, fibrin or anti-fibronectin.

In such diagnostic kit according to the invention the function of the detection component may be based on enzymatic activity, radiation emitted by a radioactive isotope or fluorescence.

The invention will be further illustrated in the following by non-limiting specific examples. This illustration is associated with references to the appended drawing, wherein:

Fig. 1 shows the binding of IgA, fibronectin (FN) and a complex to a heparin-Sepharose column;

Fig. 2 shows a diagram on the elution of proteins from an ordinary ion exchanger using a salt gradient;

Fig. 3 shows the binding of IgA, fibronectin and a complex to a jacalin-Sepharose column; and

Fig. 4 shows the separation of proteins using SDS-PAGE and immunoblotting.

EXAMPLE 1

Antisera. Patient sera were from patients with primary IgA nephropathy (glomerulonephritis and dominating mesangial IgA deposits detected by immunofluorescense microscopy of renal biopsy specimens). None of the patients had signs of systemic lupus erythematosus (SLE), Henoch-Schönleins purpura or cirrhotic liver disease. Sera from healthy blood donors were used as controls.

EXAMPLE 2

Isolation of immune complexes. Antisera (1 ml) were dialysed against 0.05 M Tris, 0.05% sodium azide pH 7.4 before applied to a 10 ml Heparin-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden). Material eluted from the column with 0.5 M NaCl in the same buffer was dialysed against 0.1 M sodium phosphate, 0.05% sodium azide pH 7.5 before chromatographed

on a 1 ml Mono Q column (Pharmacia). Chromatography was performed using a LKB (Bromma, Sweden) HPLC system with low pressure mixing. Absorbance was measured at 280 nm with a LKB 2151 variable wavelength detector. Fractions binding to collagen I and reacting with anti human IgA (Dakopatts, Hägersten, Sweden) were pooled and applied to a jacaline agarose (Pierce chemicals, Rockford, Illinois). IgA containing complexes were eluted with 0.2 M melibiose in a buffer containing 0.05 M Tris, 0.15 M NaCl, 0.05% sodium azide pH 7.5.

EXAMPLE 3

Preparation of collagen I. Collagen I was prepared by pepsin extraction from bovine flexor tendon as described by Vogel et al (12).

EXAMPLE 4

Electrophoresis. SDS-PAGE was performed as described by Laemmli (13) using 3-16% linear gradient gels. Gels were stained with silver according to the method of Morrisey (14) omitting glutaraldehyde. Reduction was performed by adding 2-mercaptoethanol to 0.2% v/v to samples prior to electrophoresis and boiling for two minutes.

EXAMPLE 5

ELISA. Antigen was coated to polystyrene 96-well microtiter plates (NUNC immunoplate I, NUNC, Roskilde, Denmark). Fractions from the columns were coated over night under non denaturing conditions using 0.05 M sodium carbonate buffer at pH 9.6 containing 0.05% sodium azide. This was followed by incubation for 1 hour with the same buffer also containg 2% bovine serum albumin (blocking buffer) to prevent non specific binding. Collagen I was coated under denaturing conditions using 6 M guanidine-HCl , 0.05 M Tris-HCl pH 7.4 over night. In this case incubation with blocking buffer was not needed. Sera were diluted in 0.01 M

phosphate pH 7.5, 0.15 M NaCl, 0.05% Tween 20 and 0.25 M guanidine-HCl and incubated for 1 hour in the coated microtiter plate. IgA antibodies were detected by incubation with affinity purified anti human IgA alkaline phosphatase conjugate (Dako) for one hour. Fibronectin was detected using rabbit anti human fibronectin antiserum (Dako) followed by anti rabbit-IgG alkaline phophatase conjugate (Dako). Enzyme activity was determined using p-nitrophenyl phosphate as the substrate. Microtiter plates were rinsed between each step with 0.15 M NaCl containing 0.05% Tween 20. All samples were analysed as triplicates. Absorbance was monitored at 405 nm using a Titertek Multiskan photometer.

EXAMPLE 6

Immunoblotting. Proteins were separated using SDS-PAGE and electrophoretically transferred to nitrocellulose paper (Schleicher and Schüll,-Dassel,W.Germany) at 0.5 A for 4 hours. Non specific binding was prevented by incubation with blocking buffer for 1 hour. IgA antibodies were detected using peroxidase conjugated anti human IgA antiserum (Dako) and fibronectin was detected using rabbit anti human fibronectin followed by peroxidase conjugated anti rabbit antiserum. Enzyme activity was determined with $H_2O_2$/diaminobenzidine (0.5mg/ml) (Fluka) in 0.05 M sodium phosphate pH 7.5 containing cobalt chloride and ammonium nickel sulfate (15).

EXAMPLE 7

Isolation of CNBr-fragments. Since we have previously found that IgA antibodies from patients with IgA nephropathy bind to collagen I,II and IV (1) an attempt was made to isolate the specific epitope of bovine collagen I responsible for the binding. Pepsin extracted collagen I was fragmented by the use of CNBr and the fragments were separated by cation exchange chromatography on a Mono S column followed by gel chromatograp-

hy on a TSK G3000SW column. Binding of antibodies to collagen I could be completely inhibited by fragments identified as the CB7 fragment of the alfa$_1$ chain and the CB3,5 fragment of the alfa$_2$ chain.

These fragments are known to contain the fibronectin binding domain (16). Thus, it appears that fibronectin is involved in the binding of IgA antibodies to collagen. Indeed, as we found for patient IgA antibodies, Engvall et al (8) have previously shown that fibronectin binds to both collagens I and II and that binding is enhanced when denatured collagens are used. In, further support we could demonstrate by ELISA that anti fibronectin antibodies coated to a microtiter plate caused binding of IgA antibodies as well as fibronectin when incubated with serum from patients with primary IgA nephropathy.

When using serum from healthy blood donors as control no IgA antibodies were bound to the anti fibronectin antibody coat.
We therefore decided to purify the complexes, in principle using affinity columns for fibronectin and IgA, respectively, in sequence.

EXAMPLE 8

Adsorption of fibronectin to heparin-Sepharose. Heparin-Sepharose (9) was used as the initial step for the purification of fibronectin-IgA complexes from human serum. As demonstrated in ELISA (fig I), using the fractions as coat, expectedly most of the IgA antibodies were not retained on the heparin column. The fibronectin bound to the heparin column, and was eluted using 0.5 M NaCl (fig 1).

It is known that fibronectin binds to collagen by its collagen binding domain located in the N-terminal part of the molecule (7,17). The collagen binding properties of fibronectin were utilized in an ELISA to demonstrate the presence of fibronectin-IgA immune complexes in the eluate from the heparin column. Denatured collagen I (17) was used as

coat. Fractions from the heparin-Sepharose column were incubated in the collagen coated microtiter wells. Bound IgA antibodies were detected using a specific antibody conjugate (fig 1). Only the fraction eluted from the heparin column by the use of NaCl contained antibody reactivity in the assay, apparently representing fibronectin-IgA immune complexes in which the IgA antibodies are bound to fibronectin and fibronectin mediates binding to collagen by its collagen binding domain.

The major portion of the IgA antibodies did not bind to the heparin sepharose and no collagen binding IgA antibodies were found in this unbound fraction.

Contrasting results were obtained with a serum sample from a healthy blood donor. No immune complexes were detected neither in the fraction bound to the heparin-Sepharose nor in the unbound fraction (fig 1).

EXAMPLE 9

Binding of immune complexes to anion exchange column. Since heparin binds not only fibronectin, but other plasma proteins as well (9) an ion exchange column was selected for the next step in the purification procedure. Material eluted from the heparin column was applied to a Mono Q, anion exchange, HPLC column. Immune complexes, detected by the ELISA with collagen I coat, were eluted between 0.22-0.24 M NaCl (fig 2). The presence of free IgA antibodies (separated from the immune complexes) demonstrates that a small proportion of the IgA binds to the heparin column and/or IgA is liberated due to dissociation of immune complexes during the purification procedure.

EXAMPLE 10

Adsorption of the IgA component in immune complexes to jacalin-Sepharose. Fractions containing immune complexes (fig 2) were pooled and chromatographed on a jacalin-Sepharose column. Jacalin is a lectin that

can be used for binding of IgA antibodies (18). This provides a possibility to separate the complexes from free fibronectin since this structure is not present on the fibronectin molecule. As expected fibronectin is found both in the material not bound to the jacalin column and in the bound fraction that was eluted with melibiose, while IgA antibodies are present only in the bound material (fig 3). The fibronectin component of the immune complexes was bound to collagen I in an ELISA and fibronectin and IgA antibodies were identified (fig 3). The presence of components with mobilities corresponding to fibronectin and IgA in the material bound to the jacalin column was further demonstrated with SDS-PAGE both with and without reduction (fig 4). Further proof for the identity of the components was obtained by immunoblotting using anti-IgA and anti-fibronektin respectively (fig 4). The major components visualized by SDS-PAGE was thus shown to represent IgA and fibronectin,respectively.

The above examples show that patients with IgA nephropathy have circulating immune complexes containing fibronectin and IgA antibodies. This is in agreement with a proposed immune complex mediated nature of primary IgA neprhopathy (11).

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CLAIMS

1. A method for the diagnosis of IgA nephropathy using an antigene-antibody interaction, characterized by the steps:
a) preparing a substrate capable of binding fibronectin or IgA
b) contacting the substrate resulting from step a) with a sample of body fluid drawn from a patient subject to diagnosis to bind any fibronectin-IgA-complex present in said sample tc the substrate, and
c) determining the presence of complex bound to the substrate using the reaction between the exposed part of such bound complex and a corresponding antibody thereto.

2. A method according to claim 1, characterized by preparing a substrate capable of binding via collagen, fibrin or heparin to the fibronectin part of the complex.

3. A method according to claim 1, characterized by preparing a substrate capable of binding via anti-fibronectin to the fibronectin part of the complex.

4. A method according to claim 1, characterized by preparing a substrate capable of binding via anti-IgA or lectin to the IgA-part of the complex.

5. A method according to any preceding claim, wherein the body fluid is constituted by human blood, serum, plasma or saliva.

6. A method according to any preceding claim, charactertized in that in step c) the presence of bound complex is measured quantitatively using a detection system based on enzymatic activity, radiation emitted by a radioactive isotope or fluorescence.

7. A method according to claim 2 or 3, characterized by using under step c) the reaction between the IgA-part of the complex and an anti-IgA- or lectin-enzyme complex.

8. A method according to claim 4, characterized by using under step c) the reaction between the fibronectin part of the complex and an anti-fibronectin-, heparin- , fibrin- or collagen-enzyme complex.

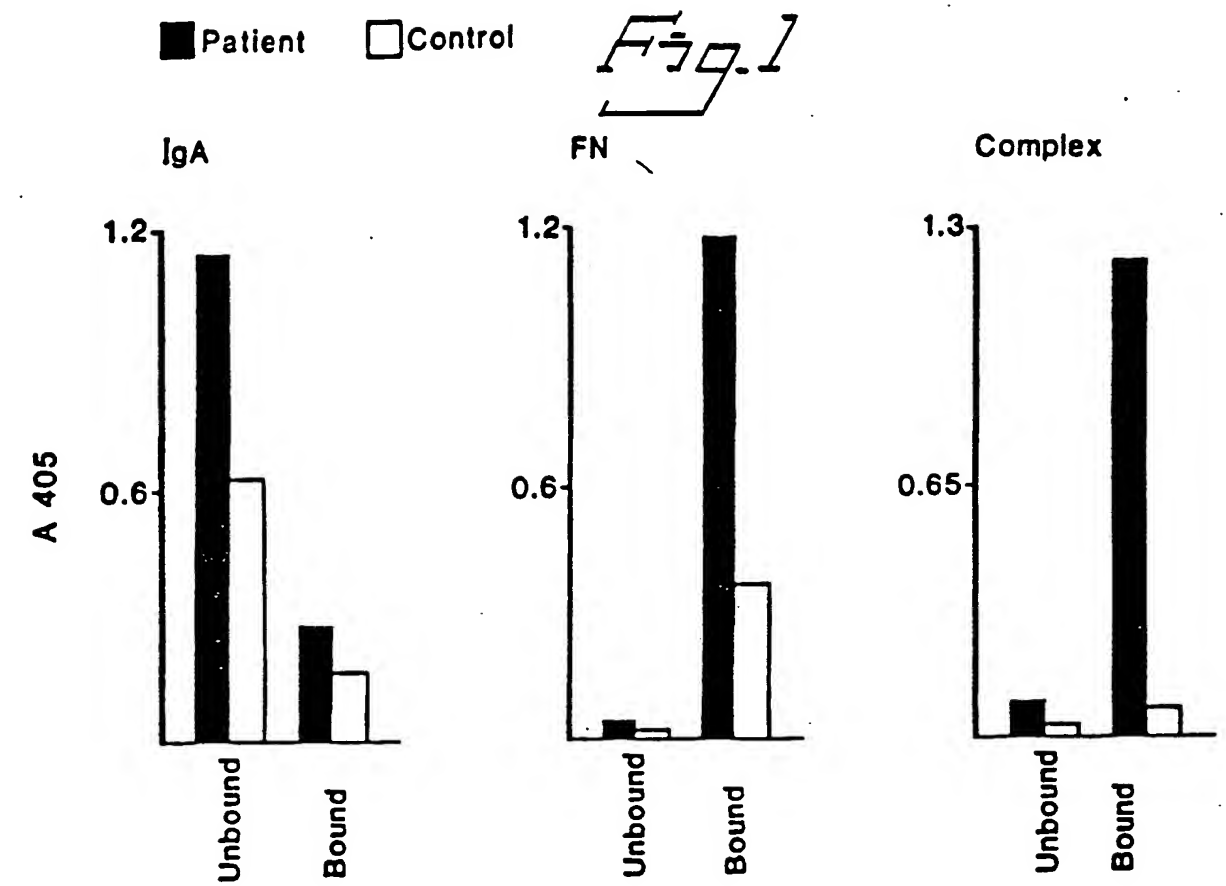
9. A method according to any preceding claim, wherein the substrate is constituted by a microtiter plate.

10. A diagnostic kit for use in the diagnosis of IgA nephropathy, comprising:
a) an object capable of binding fibronectin or IgA, and
b) a reagent comprising a component capable of binding IgA or fibronectin and a detection component enabling determination of fibronectin-IgA complex bound to the object.

11. A diagnostic kit according to claim 10, wherein the object carries attached to the surface thereof heparin, collagen, fibrin or anti-fibronectin, and wherein the reagent comprises lectin or antibody directed against IgA.

12. A diagnostic kit according to claim 10, wherein the object carries attached to the surface thereof anti-IgA or lectin, and wherein the reagent comprises heparin, collagen, fibrin or anti-fibronectin.

13. A diagnostic kit according to claim 10 or 11, wherein the function of the detection component is based on enzymatic activity, radiation emitted by a radioactive isotope or fluorescence.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FN
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Unbound
Bound
Unbound
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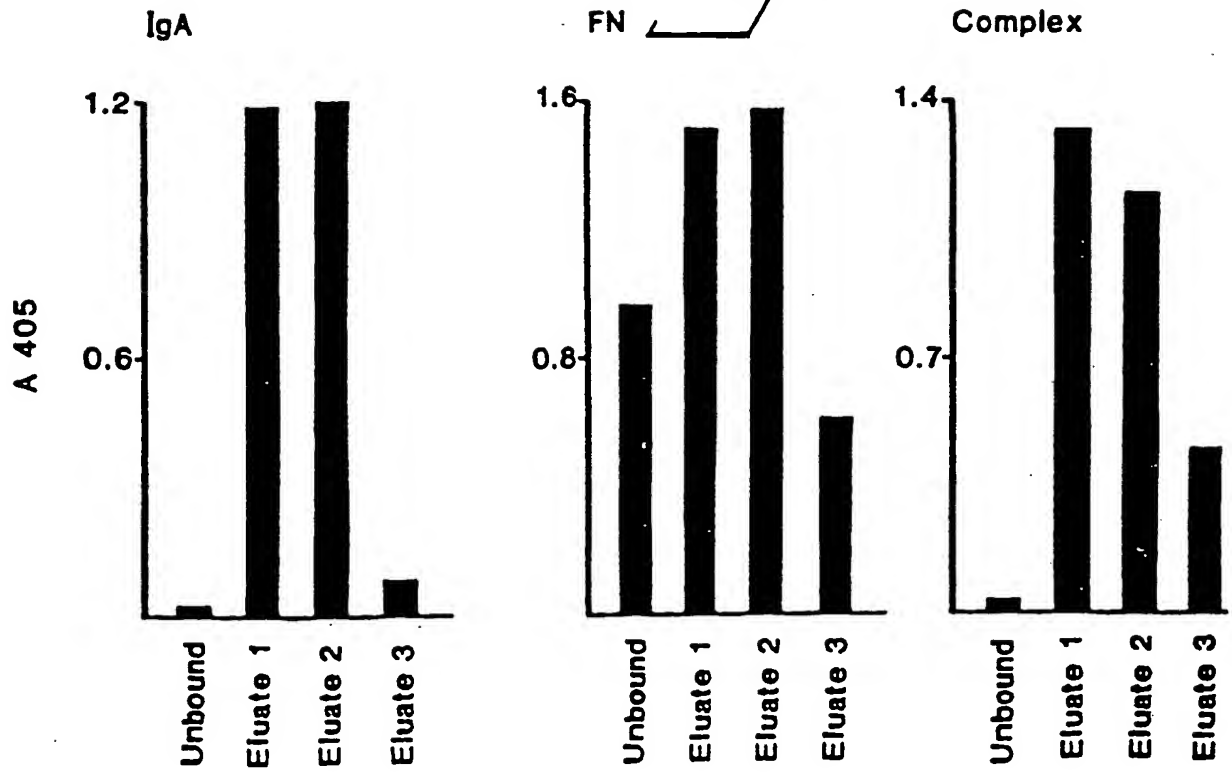
Bound

Fig. 1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Unbound
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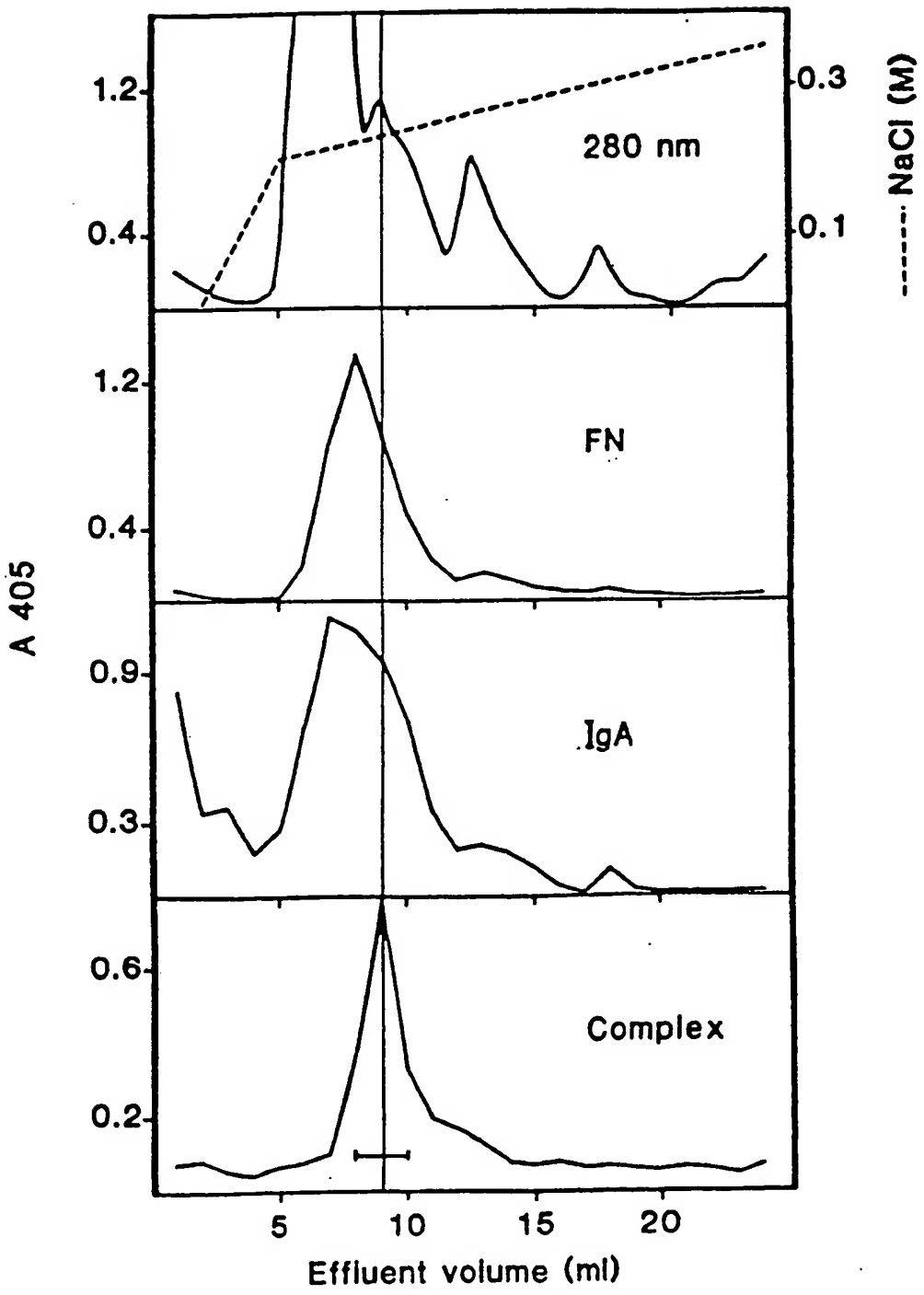
Eluate 3

Fig. 3

Fig. 2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280 nm
0.3
0.1
NaCl (M)
1.2
0.4
FN
A 405
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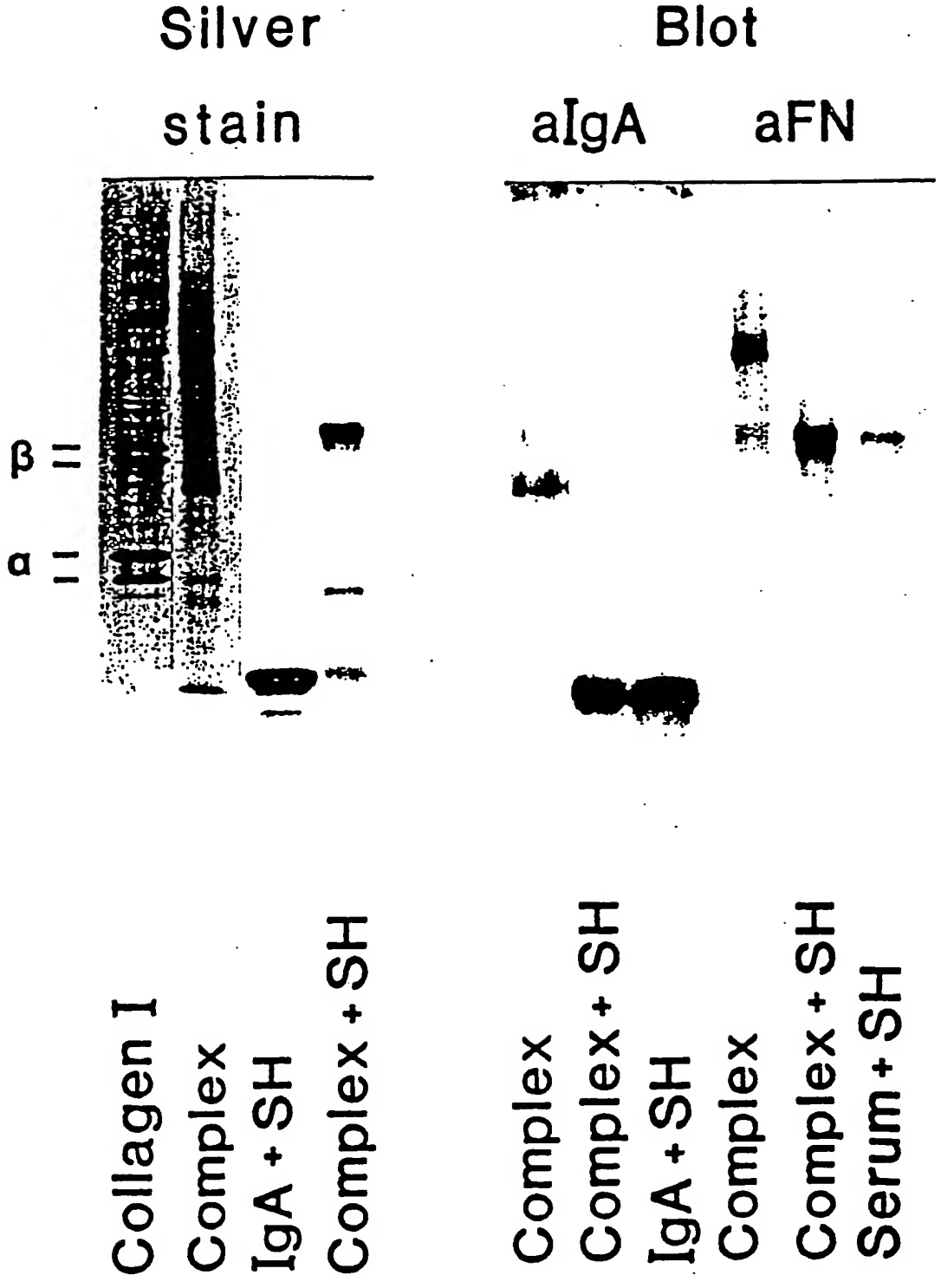
0.3
IgA
0.6
0.2
Complex
5
10
15
20
Effluent volume (ml)

Fig. 4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stain
Blot
aIgA
aFN
β
α
Collagen I
Complex
IgA + SH
Complex + SH
Complex
Complex + SH
IgA + SH
Complex
Complex + SH
Serum + SH